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An Amidolytic Assay for Determination of α_1-Antitrypsin in Serum and in Cerebrospinal Fluid

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Summary: An amidolytic assay using the chromogenic substrate tosyl-glycyl-prolyl-lysine-4-nitranilide acetate (Chromozym PL®) has been studied for the determination of α_1-antitrypsin in serum and in cerebrospinal fluid (CSF). It was found that binding of trypsin to α_2-macroglobulin has to be taken into consideration when α_1-antitrypsin is determined by an amidolytic assay. In serum this accounts for 7.4 ± 3.2% and in CSF 2.8 ± 1.8% of the total antitrypsin capacity. The reference range of α_1-antitrypsin in sera was found to be $\bar{x}$ = 60.3 ± 20.8 kIU/l, and in CSF $\bar{x}$ = 186 ± 99 IU/l. Within-run precision showed a C.V. of 1.36% in sera, and a C.V. of 1.86% in CSF. There was a good correlation with immunochemical methods: r = 0.946 in 65 sera ($p < 0.001$) and r = 0.986 in 55 samples of CSF ($p < 0.001$).

Bestimmung von α_1-Antitrypsin in Serum und Liquor mit einem chromogenen Substrat

Zusammenfassung: Es wurde ein photometrisches Verfahren mit dem chromogenen Substrat Tosyl-glycyl-prolyl-lysin-4-nitranilidacetat (Chromozym PL®) zur Bestimmung des α_1-Antitrypsingehaltes im Serum und im Liquor untersucht. Dabei ist bei der Bestimmung des α_1-Antitrypsins die Bindung von Trypsin an α_2-Makroglobulin zu berücksichtigen. Im Serum wurde dafür ein Anteil von 7,4 ± 3,2% und im Liquor von 2,8 ± 1,8% an der gesamten Antitrypsinkapazität ermittelt. Als Referenzbereich von α_1-Antitrypsin wurden folgende Werte gefunden: im Serum $\bar{x}$ = 60,3 ± 20,8 kIU/l und im Liquor $\bar{x}$ = 186 ± 99 IU/l. Für die Präzision in der Serie wurde im Serum ein VK von 1,36 und im Liquor von 1,86% erhalten. Weiterhin bestand eine ausgezeichnete Übereinstimmung zu immunchemischen Methoden: r = 0,946 in 65 Seren ($p < 0,001$) sowie r = 0,986 in 55 Liquorproben ($p < 0,001$).

Introduction

Evaluation of the serum-CSF barrier gradient of α_1-antitrypsin has received increasing attention within recent years (2, 3). However, the determination of α_1-antitrypsin in serum with an amidolytic assay provides several problems. It is known from the literature that an increase in the dilution of serum or plasma results in a greater inhibition of trypsin activity (4). A dilution of one hundred times exhibits the largest degree of inhibition. Further dilution of more than five hundred times leads to a marked decrease in the inhibition of trypsin activity (4). The development of a number of chromogenic substrates has provided a substrate for the determination of α_1-antitrypsin according to the above conditions. Moreover, cerebrospinal fluid (CSF) should be assayed without a preparation step prior to the photometric assay. It is well established that trypsin also binds to other antiproteases, like α_2-macroglobulin. Inter-α-trypsin-inhibitor exhibits a lower affinity to trypsin than other antiproteases and is therefore of minor importance (5). However, the influence of α_2-macroglobulin on the assay has to beconsidered. The reaction of the assay can be scheduled as follows:

1a. Trypsin + α_1-antitrypsin $\rightleftharpoons$ trypsin-α_1-antitrypsin complex (inactive) + trypsin (free, active)

1b. Trypsin + α_2-macroglobulin $\rightleftharpoons$ trypsin-α_2 macroglobulin complex (active) + trypsin (free, active)

Trypsin (free, active) + STI[2]) $\rightleftharpoons$ trypsin-STI complex (inactive)

2. Chromozym PL® $\xrightleftharpoons{\text{trypsin (active)}}$ peptide + 4-nitroaniline

(active trypsin = free trypsin + trypsin-α_2-macroglobulin complex)

It was the purpose of this study to evaluate optimal conditions for such an amidolytic assay.

[1]) Presented in part at the XI. International/IV. European Congress of Clinical Chemistry, Vienna, Austria, Sept. 1981 (1)

[2]) STI: soybean trypsin inhibitor

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Materials and Methods

Samples

65 sera and 55 samples of cerebrospinal fluid, representing unselected clinical material which had been sent to our laboratory, were investigated. For determination of kinetic parameters, within-run, and day to day precision a pool serum of 20 healthy donors and a CSF pool of 20 samples selected at random were taken. All samples were immediately frozen at − 20 °C and analyzed within 4 weeks. α_1-antitrypsin is known to remain stable within this period (6).

Prior to the photometric assay, sera were diluted 1 + 100 with a solution of 9 g/l of sodium chloride.

Reagents and procedures

Protein was measured in sera and in CSF by standard procedures (7, 8). Cell count in CSF was estimated from a microscopic count. Immunochemical levels of α_1-antitrypsin were calculated from immunodiffusion (NOR-Partigen and LC-Partigen, Behring Institute Marburg, F.R.G.) (9). For the amidolytic assay, the reagents were commercial preparations of analytical grade, purchased from Boehringer Mannheim, F.R.G. (Chromozym TH®, Chromozym TRY®, and Chromozym PL®, trypsin (33 U/mg protein with benzoyl-*D,L*-arginine-4-nitroanilide as substrate), and soybean trypsin inhibitor (STI)). The buffer consisted of 0.05 mol/l of triethanolamine and 0.15 mol/l of sodium chloride, adjusted to pH 8.0. The following CSF values were considered normal: protein level below 500 mg/l, cell count below 3 cells/mm^3, no red cells on microscopic examination. For the calculation of the serum/CSF barrier gradient, sera exhibiting α_1-antitrypsin levels below 2 g/l and more than 4 g/l were excluded. K_m-values were calculated from *Eadie-Hofstee* plots (10, 11).

Results

Methodological studies

The chromogenic substrates Chromozym TH®, Chromozym TRY®, and Chromozym PL® were evaluated for determination of α_1-antitrypsin in serum. The substrates Chromozym TH® and Chromozym TRY®, both very sensitive to trypsin, require a dilution of sera of at least 600 fold; the substrate Chromozym PL®, however, required only a dilution of one hundred fold (high levels of α_1-antitrypsin in serum require a 200 fold dilution) and corresponded to the conditions mentioned above. This dilution resulted in a change of absorbance of $\Delta A/min = 0.4$ for the chromogenic substrate Chromozym PL®; for the substrates Chromozym TH® and TRY®, however, a change of absorbance of $\Delta A/min = 2.6$ was observed.

A linear slope function representing the splitting of the substrate Chromozym PL® by trypsin was obtained over a wide range. A change of absorbance of $\Delta A/min = 0.350$ corresponded to a final concentration of 25 U/l trypsin. In further experiments it was revealed that the substrate Chromozym PL® is split by the trypsin-α_2-macroglobulin complex, as already described for other substrates (12, 13).

Kinetics of inhibition of the amidolytic activity of trypsin by α_1-antitrypsin were evaluated at 25 °C and 37 °C (fig. 1). It is evident that trypsin reacts slowly with α_1-antitrypsin: half-rate at 25 °C was determined to be 142 seconds and at 37 °C to be 59 seconds (fig. 1).

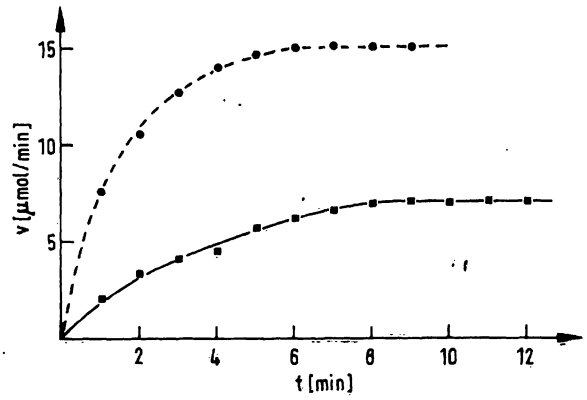

Fig. 1. Variation of the incubation time of trypsin with a pre-diluted serum at 25 °C (□) and at 37 °C (●) (standard conditions). Prolongation of incubation leads to a decrease of free trypsin.

Stepwise addition of diluted serum leads to a non-competitive inhibition of the amidolytic activity of trypsin (fig. 2). For the splitting of the chromogenic substrate Chromozym PL® by trypsin, the K_m was 2×10^{-5} mol/l, independent of the amount of diluted serum in the assay.

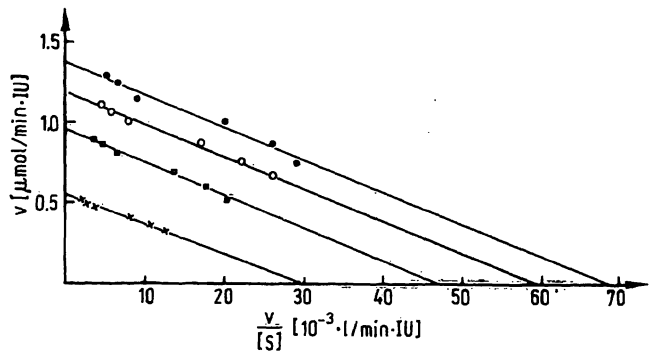

Fig. 2. Non-competitive inhibition of the amidolytic activity of trypsin by stepwise addition of diluted pool serum (standard conditions) (●: no addition of diluted serum, ○: addition of pool serum, diluted 1:400, ■: addition of pool serum, diluted 1:200, ×: addition of pool serum, diluted 1:100; IU: amount of α_1-antitrypsin in Inhibitory Units).

Tab. 1. Contribution of α_2-macroglobulin to the total antitrypsin capacity, depending on the α_1-antitrypsin levels in serum and in CSF.

Level of antitrypsin in serum	% of the total antitrypsin capacity due to α_2-macroglobulin
< 2.5 g/l (n = 19)	13.5 ± 9.2
2.5–4.0 g/l (n = 28)	7.7 ± 2.6
> 4.0 g/l (n = 18)	4.2 ± 1.8
Total (n = 65)	7.4 ± 3.2
Levels of antitrypsin in CSF	**% of the total antitrypsin capacity due to α_2-macroglobulin**
< 20 mg/l (n = 32)	3.4 ± 1.7
20–50 mg/l (n = 14)	1.8 ± 0.9
> 50 mg/l (n = 9)	2.5 ± 1.9
Total (n = 55)	2.8 ± 1.8

In simultaneous assays, the contribution by α_2-macroglobulin to the total antitrypsin amount was determined as previously described (12, 13), using the ability of α_2-macroglobulin to form a trypsin-protein complex, which retains its amidolytic activity, also in the presence of soybean trypsin inhibitor. These results are summarized in table 1. It is evident that in the case of a low α_1-antitrypsin level, a considerable part of trypsin will be bound to α_2-macroglobulin.

All this considered, the photometric assays can be summarized as follows:

assay a)

0.40 ml of buffer with trypsin (25 U/l)
0.02 ml of sample (serum dilution 1 + 100 or CSF)
6 min incubation
0.05 ml of Chromozym PL® (3.2×10^{-4} mol/l)
increase in absorbance at 405 nm; d = 1 cm, 37 °C;

assay b)

0.40 ml of buffer with trypsin (25 U/l)
0.02 ml of sample (serum dilution 1 + 100 or CSF)
1 min incubation
0.01 ml soybean trypsin inhibitor (5×10^{-7} mol/l)
0.05 ml of Chromozym PL® (3.2×10^{-4} mol/l)
increase in absorbance at 405 nm; d = 1 cm, 37 °C.

Calculation was performed as follows:

$$\Delta A/min_{trypsin} - \Delta A/min_{sample\ (assay\ a)} - \Delta A/min_{\alpha_2\text{-macroglobulin (assay b)}} = \Delta A/min_{trypsin\ inhibited\ by\ \alpha_1\text{-antitrypsin}}$$

Using a pool serum the assay revealed a good linearity over a wide range (fig. 3). In the case of serum, the assay should be carried out in the range from 400 to 800 IU/l[3]) (sera should be diluted 100 fold or 200 fold, respectively); evaluation of the CSF levels, however, covers the total range.

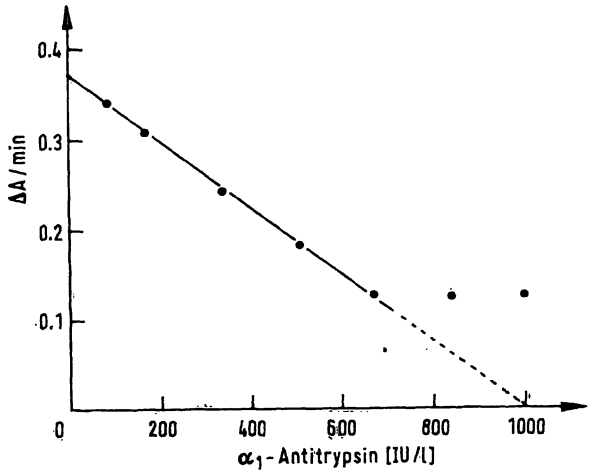

Fig. 3. Linearity of the assay, obtained with a pool serum (standard conditions; IU: amount of α_1-antitrypsin in Inhibitory Units).

[3]) 1 IU = 1 Inhibitor Unit = the amount of α_1-antitrypsin, which inhibits 1 U (µmol/min) of trypsin (Chromozym PL® as the substrate)

As the CSF samples were taken without dilution, the unspecific splitting of the chromogenic substrate was evaluated in the CSF samples: even in the pathological range unspecific splitting did not exceed 0.5% of total splitting.

Reference range

With the assay, the normal range of α_1-antitrypsin in sera was found to be 60.3 ± 20.8 kIU/l (n = 65) (total protein concentration 72 ± 9.2 g/l). In CSF, the reference range was 186 ± 99 IU/l (n = 55) (total protein did not exceed 500 mg/l); no sex differences were observed.

Within-run precision and detection limit

Within-run precision was $\bar{x}$ = 52.3 ± 0.71 kIU/l (C.V. = 1.36%) for pool serum and $\bar{x}$ = 326 ± 6 IU/l (C.V. = 1.86%) for a CSF pool. Detection limit in CSF was 12 IU/l.

Day to day precision

For serum, day to day precision was found to be $\bar{x}$ = 55.1 ± 1.53 kIU/l (C.V. = 2.78%) and for CSF $\bar{x}$ = 319 ± 11 IU/l (C.V. = 3.45%).

Correlation with immunodiffusion

There was a good correlation with immunodiffusion in sera as well as in CSF (r = 0.946 for 65 sera ($p < 0.001$), and r = 0.986 for 50 samples of CSF ($p < 0.001$), fig. 4 and 5). Linear regression analysis showed the equation $y = 0.054\ x + 0.05$ for sera and $y = 0.070\ x - 3.2$ for CSF. 5 samples of CSF had to be excluded because of their low α_1-antitrypsin levels (not detectable with immunodiffusion).

Serum-CSF barrier gradient

Using the sera that exhibited an α_1-antitrypsin level from 2 to 4 g/l and the CSF samples that were considered normal, the serum-CSF barrier gradient was calculated. Using the amidolytic assay, a gradient of 276 was found, while immunodiffusion gave a value of 269.

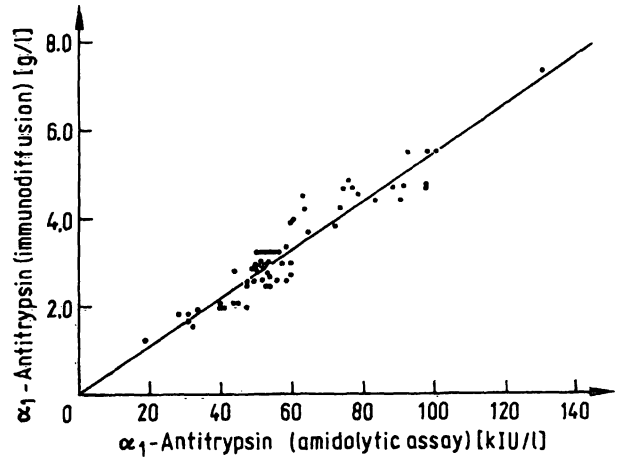

Fig. 4. Comparison of the amidolytic assay of α_1-antitrypsin with immunodiffusion in 65 sera at random ($y = 0.054\ x + 0.05$, $r = 0.946$, $p < 0.001$; IU: amount of α_1-antitrypsin in Inhibitory Units).

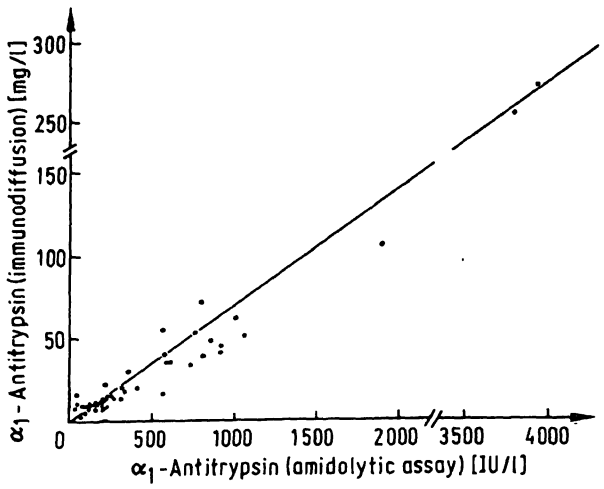

Fig. 5. Comparison of the amidolytic assay of α_1-antitrypsin with immunodiffusion in 50 CSF samples at random ($y = 0.070\ x - 3.2$, $r = 0.986$, $p < 0.001$; IU: amount of α_1-antitrypsin in Inhibitory Units).

Discussion

It is well known that α_1-antitrypsin can be regarded the major inhibitor of trypsin in serum. However, the amount of trypsin bound to α_2-macroglobulin is variable. Consequently, an exact determination of α_1-antitrypsin using a functional assay requires exploration of the α_2-macroglobulin level. On the other hand, in CSF the low levels of $\alpha_2$macroglobulin are negligible.

It is well known that the chromogenic substrate Chromozym PL® is also split by other serine proteases like plasmin. As plasmin generally does not occur as a free protease in plasma or serum, an unspecific splitting of the chromogenic substrate by free plasmin can be ruled out.

Our results suggest a serum-CSF barrier gradient of ca. 270 for α_1-antitrypsin, which agrees with the findings of *Felgenhauer* (14).

It has already been reported that α_1-antitrypsin reacts slowly with trypsin (4). In a previous study, we demonstrated that trypsin reacts rapidly with α_2-macroglobulin (13). Up to now, the understanding of the physiological relevance of these observations is very poor. *Takada* et al. (4) assumed that binding of trypsin to α_2-macroglobulin may have a protective effect, thus preserving the efficacy of the proteinase in breaking down physiologically active substances such as peptide hormones.

Two recent reports have pointed to the diagnostic relevance of the determination of α_1-antitrypsin in CSF. In patients with intracranial tumours, raised levels could be observed (3). On the other hand, markedly lowered levels were reported in patients suffering from multiple sclerosis (2). It is interesting that in the case of multiple sclerosis, serum levels remain unaffected, while the decrease of the α_1-antitrypsin level in CSF is evident (2).

These findings suggest that in these cases α_1-antitrypsin is involved in the local immune response. Data, recently presented by *Arora* et al (15), indicate that α_1-antitrypsin is an effector of immunological stasis. α_1-antitrypsin was found to suppress the in-vitro and in-vivo immune response: increasing amounts of α_1-antitrypsin resulted in even greater suppression of immune response (15).

All these considerations make it apparent that a determination of α_1-antitrypsin can be used with advantage in the diagnosis of various neurological diseases. As lowered levels of α_1-antitrypsin in CSF cannot be exactly quantified by immunodiffusion, an amidolytic assay might be useful.

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